I hereby certify that this correspondence is being electronically filed in the United States Patent and Trademark Office on 3 June 2009.

Jeff Lloyd, Patent Attorney, Reg. No. 35,589

REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 CFR 1.322 AND UNDER 37 CFR 1.323
Docket No. UF.155CD3
Patent No. 7,485,771

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | |
|---|---|---|
| Applicants | : | Robert R. Schmidt, Philip Miller |
| Issued | : | February 3, 2009 |
| Patent No. | : | 7,485,771 |
| Conf. No. | : | 5539 |
| For | : | Polypeptides and Polynucleotides Relating to the Alpha- and Beta-Subunits of Glutamate Dehydrogenases and Methods of Use |

Mail Stop Certificate of Corrections Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION
UNDER 37 CFR 1.322 (OFFICE MISTAKE)
UNDER 37 CFR 1.323 (APPLICANT MISTAKE)

Sir:

A Certificate of Correction for the above-identified patent has been prepared and is attached hereto.

In the left-hand column below is the column and line number where errors occurred in the patent. In the right-hand column is the page and line number in the application where the correct information appears.

| **Patent Reads:** | **Application Should Read:** |
|---|---|
| Column 6, lines 50-51: | Page 10, line 11: |
| "glutamate the precursor to" | --glutamate, the precursor to-- |
| Column 7, line 30: | Page 11, line 14: |
| "substantially that found in plants" | --substantially found in plants-- |
| Column 9, lines 66-67: | Page 15, line 11: |
| "regeneration in to whole plants" | --regeneration into whole plants-- |
| **Patent Reads:** | **Application Reads:** |
| Column 11, line 28: | Page 17, line 15: |
| "NADP+" | --$NADP^+$-- |
| **Patent Reads:** | **Application Should Read:** |
| Column 12, line 35: | Page 19, line 14: |
| "values determine for" | --values determined for-- |
| Column 13, line 44, Table 3: | Page 21, line 12, Table 3: |
| "240 1.12 34.79" | --240 1.13 34.79-- |
| Column 14, line 43: | Page 22, line 25: |
| "after a 3 minutes" | --after 3 minutes-- |
| **Patent Reads:** | **Application Reads:** |
| Column 14, line 52: | Page 23, line 2: |
| "2xLiCl-Urea" | --2X LiCl-Urea-- |
| **Patent Reads:** | **Application Should Read:** |
| Column 16, line 45: | Page 25, line 30: |
| "gel agarose gel purified" | --gel agarose, gel purified-- |

| **Patent Reads:** | **Application Reads:** |
|---|---|
| Column 17, line 8: | Page 26, line 20: |
| "P-GDH" | --β-GDH-- |
| Column 18, line 64: | Page 29, line 18: |
| "11 a-α-cDNA" | --11a-α-cDNA-- |
| Column 59, line 59: | Page 33, original claim 7, renumbered as claim 2: |
| "a-GDH or 13 -GDH" | --α-GDH or β-GDH-- |

A true and correct copy of pages 17, 23, 26, 29, and 33 of the specification as filed which support Applicants' assertion of the errors on the part of the Patent Office accompanies this Certificate of Correction.

The fee of $100.00 was paid at the time this Request was filed. The Commissioner is also authorized to charge any additional fees as required under 37 CFR 1.20(a) to Deposit Account No. 19-0065.

Approval of the Certificate of Correction is respectfully requested.

Respectfully submitted,

Jeff Lloyd
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JL/abt
Attachments: Copies of pages 17, 23, 26, 29, and 33 of the specification
Certificate of Correction

**UNITED STATES PATENT AND TRADEMARK OFFICE**

# CERTIFICATE OF CORRECTION

PATENT NO. : 7,485,771 Page 1 of 2

APPLICATION NO.: 10/627,886

ISSUE DATE : February 3, 2009

INVENTORS : Robert R. Schmidt, Philip Miller

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 6,
Lines 50-51, "glutamate the precursor to" should read --glutamate, the precursor to--.

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Column 14,
Line 43, "after a 3 minutes" should read --after 3 minutes--.
Line 52, "2xLiCl-Urea" should read --2X LiCl-Urea--.

MAILING ADDRESS OF SENDER:
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This collection of information is required by 37 CFR 1.322, 1.323, and 1.324. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1.0 hour to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending on the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Attention Certificate of Corrections Branch, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

# UNITED STATES PATENT AND TRADEMARK OFFICE
# CERTIFICATE OF CORRECTION

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Purification of the NADP-GDH isoenzymes. For purification of the glutamate dehydrogenase α-isoenzyme, *C. sorokiniana* cells were cultured with continuous light in 29 mM ammonium medium in a 30 L Plexiglas chamber as previously described (Baker, A.L., R.R. Schmidt [1963] *Biochim. Biophys. Acta* 74:75-83). Cells were harvested at 4.0 $OD_{640}$ by centrifugation at 30,000 rpm through a Sharples centrifuge and washed two times in 10 mM Tris (pH 8.5 at 4°C). Pelleted cells (130 g) were stored at –20°C in 250 mL centrifuge bottles until use. Purification of NADP-GDH was accomplished using a modified procedure of Yeung *et al., supra.* Procedural modifications involved the substitution of Sephadex G-200 gel (Pharmacia) for G-150 gel in the gel-filtration column, and the addition of $NADP^+$ as a stabilizer to a final concentration of 0.1 mM to the gel-filtration buffer and all subsequent storage buffers. As a final modification, the $NADP^+$ affinity resin step was omitted and a preparative nondenaturing-PAGE step was substituted (Miller, P.W., W.D. Dunn, R.R. Schmidt [1994] *BioRad US/EG Bulletin 1897*).

The GDH deaminating enzyme assay solution was composed of 44 mM Tris, 20.4 mM glutamate, and 1.02 mM $NADP^+$, pH 8.8. The aminating assay solution was composed of 50 mM Tris, 25 mM α-ketoglutarate, 0.357 mM NADPH, and 0.356 M $(NH_4)_2SO_4$, pH 7.4. One unit of enzyme activity was the amount of NADP-GDH required to reduce or to oxidize 1.0 μmol of $NADP^+$ or NADPH per minute at 38.5°C.

Sephadex G-200 column fractions possessing NADP-GDH activity were pooled and concentrated via Diaflow filtration. The soluble enzyme (68 mg) was protected from oxidation by the addition of DTT to a final concentration of 10 mM, and dialyzed for 30 minutes against 28.8 mM Tris, 192 mM glycine, 2 mM DTT (pH 8.4). The dialysate was clarified by centrifugation at 20,000*g* for 10 minutes at 4°C and was combined with 3 mL of 40% (w/v) sucrose and 1 mL of 0.02% bromophenol blue.

For preparative nondenaturing PAGE, a 3 cm tall 7% acrylamide (w/v, 28 acrylamide: 0.735 bis-acrylamide, pH 8.8) resolving gel, and a 2 cm tall 2% acrylamide (w/v, 1.6 acrylamide: 0.4 bis-acrylamide, pH 6.6) stacking gel were cast in the 28 mm ID gel tube of the Model 491 Prep Cell. All acrylamide stocks were pretreated with AG501-X8 mixed bed resin to remove any contaminating acrylic acid residue to prevent *in vitro* N-acylation

clear. After the final extraction, the aqueous phase was combined with an equal volume of 2X LiCl-Urea buffer (4 M LiCl, 4 M urea, 2 mM EDTA, 1 mM aurintricarboxylic acid; Sigma) and the RNA was precipitated on ice for 16 hours at 4°C. The RNA precipitate was centrifuged at 4000*g* for 20 minutes at 4°C and the resulting pellet was rinsed once with 1X LiCl-Urea buffer and centrifuged again to pellet the RNA. The RNA pellet was solubilized in TE (pH 7.5) and an aliquot was quantified spectrophotometrically at 260 nm. After quantitation, the mRNA fraction was isolated from total cellular RNA using an oligo(dT) spin column kit. Poly(A)$^+$ RNA (50 µg) from each preparation was combined and utilized for the commercial production of a custom λUni-ZAP XR *C. sorokiniana* cDNA library (Stratagene Cloning Systems, Palo Alto, CA).

The amplified λZAP library, containing 2 x $10^{10}$ pfu/mL, was plated on twenty 150 mm petri plates at 50,000 pfu per plate for a total of 1 x $10^6$ pfu screened. The phage plaques were absorbed to duplicate Hybond-N 132 mm circular membranes and treated according to the plaque blotting protocol of Amersham (1985, Amersham International plc, Arlington Heights, IL ). Membranes were prehybridized in a common container in 200 mL of 2X PIPES (0.8 M NaCl, 20 mM PIPES, pH 6.5), 50% (w/v) formamide, 0.5% (w/v) SDS, 100 µg/mL denatured sheared salmon sperm DNA at 40°C. Blocked membranes were hybridized at 42°C in ten heat-sealable bags (four membranes/bag) in prehybridization buffer containing 1 x $10^6$ cpm/membrane of a $^{32}$P-labeled NADP-GDH 242 bp HCR cDNA probe on a lab rocker. The membranes were washed three times in 200 mL of 0.1X SSC, 0.1% (w/v) SDS for 20 minutes per wash at 50°C. Duplicate membranes were wrapped in plastic wrap and exposed to Kodak X-Omat AR film at –70°C for 28 hours. Putative NADP-GDH cDNA plaques, detected on duplicate membranes, were cored from the plate and plaque purified by secondary and tertiary screenings with the 242 bp conserved region probe. Putative NADP-GDH cDNA phage clones, selected in the primary screening, were combined and screened a second time with a $^{32}$P-labeled 130 bp *Eco* RI/*Bgl* II cDNA fragment isolated from the 5′ terminus of the most complete 5′ end NADP-GDH cDNA clone. Ten plaque pure NADP-GDH clones were subcloned in pBluescript KS$^+$ (Stratagene) and transformed into *E. coli* DH5α F′ (Bethesda Research Laboratories, BRL) via an *in vivo* excision protocol

Expression of α- and β-homohexamers in *E. coli*. Using the gel purified product (SEQ ID NO. 18), PCR mutagenesis was performed to remove the chloroplast targeting signal from the full-length cDNA and yield cDNAs encoding specifically the mature α- and β-subunits. Two sets of primer pairs were designed to synthesize α- and β-GDH subunit genes.

The following primer was designed to add a methionine to the amino terminus of the processed mature α-NADP-GDH subunit (alanine-41) to allow translation initiation and to generate a 5′ *Nde*I site for subcloning purposes: 5′-CATATGGCCGTCTCGCTGGAGGAG-3′ (SEQ ID NO. 20). The following second primer was designed to hybridize to the 3′ terminus of the template DNA at a position 20 nt 3′ of the endogenous TAA termination codon: 5′-GTTGGATTGCCGGTGAGCC-3′ (SEQ ID NO. 21).

The following primer was designed to add a methionine to the amino terminus of the processed mature β-subunit (aspartate-38) to allow translation initiation and to generate a 5′ *Nde*I site for subcloning purposes: 5′-CATATGGACGCCACCACCGGC-3′ (SEQ ID NO. 22). The second 3′ primer used in the PCR amplification was the 3′-terminus primer (SEQ ID NO. 21) described for the α-subunit amplification.

PCR cycling conditions were as follows: 95°C, 50 seconds; 64°C, 1 minute; 72°C, 1 minute 35 seconds (30 cycles). Primer, dNTP, Vent polymerase, and other reaction component concentrations were as previously described. The 1506 bp α-NADP-GDH subunit gene (SEQ ID NO. 23) and 1473 bp β-GDH subunit gene (SEQ ID NO. 25) PCR products were gel purified and given a 3′ adenine nucleotide overhang by incubating the purified fragment with 100 μM dATP and *Taq* polymerase for 15 minutes at 72°C. The modified PCR products were cloned into the PCRII T/A cloning vector (Invitrogen) and transformed into competent *E. coli* cells. Clones bearing the inserts were selected by blue-white screening, plasmid purified, and digested with *NdeI*/*Bam*HI to select for the proper orientation in the cloning vector. The selected plasmids were restricted with *Nde*I and *Bam*HI (*Bam*HI site provided by vector) and directionally cloned under the control of the IPTG inducible T7 polymerase promoter of pET 11a and pET 15b bacterial expression vectors (Novagen) linearized with *NdeI*/*Bam*HI, and transformed into DH5α. Transformants were

cytoplasm of the cell. Such a cytosolic localized enzyme can be useful in capturing ammonium or glutamate compartmentalized in the cytosol of the cell.

GDH gene sources. The GDH gene used in the DNA constructs of the present invention can be any GDH gene. It is not limited to the *C. sorokiniana* GDH genes described above, although they are preferred. For example, a GDH gene from bacteria or fungi can be used. The examples provided use the α- and β-GDH genes of *C. sorokiniana*, but should not be interpreted in any way to limit the scope of the present invention. Individuals skilled in the art will recognize that various other genes as well as alterations can be made to genes and methods described herein while not departing from the spirit and scope of the present invention. For example, mutagenesis and routine screening can be implemented by techniques well known in the art to produce mutant variants that lack regulation by the cofactor NADPH.

Transient expression in maize protoplasts. In order to test the expression of the *C. sorokiniana* GDH subunits and their assembly into active enzymes in *Zea mays* cells, vectors were constructed to contain the CaMV E35S promoter, the coding sequence for the mature α-subunit (pMON21904) or β- subunit (pMON21905), the NOS 3′-untranslated polyadenylation region, and kanamycin resistance for selection in *E. coli*. The α- and β-subunit genes were isolated as a *Xba*I-*Eco*RI fragment from pET 11a-α-cDNA and pET 11a-β-cDNA, respectively. The GDH genes were ligated into the *Xba*I-*Eco*RI E35S promoter, NOS 3′, kanamycin resistance bearing region of pMON22072 to give pMON21904, and pMON21905. The DNA constructs were electroporated into maize and wheat protoplast according to the method of Sheen *et al.* (*The Plant Cell* Vol. 3, 225-245).

Analysis of transformed maize protoplasts. Pelleted protoplast samples transformed with pMON21904 (α-subunit), pMON21905 (β-subunit), pMON21709 (kanamycin negative control DNA), and no DNA were thawed in 0.2 mL of GDH cell breakage buffer (Yeung *et al.*, *supra*) on ice. The cells in each suspension were homogenized twice for 30 seconds, chilled on ice, and clarified at 14,000 rpm for 10 minutes. Cell extracts were assayed in the deaminating direction at 38.5°C according to Yeung *et al.*, *supra*. Total protein content of the cell extracts was determined using the BioRad microprotein assay according to the

7. The method of claim 1, wherein said polypeptide is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 24, SEQ ID NO: 26, and fragments of any of the foregoing of sufficient length to exhibit α-GDH or β-GDH activity.

8. The method of claim 1, wherein said polynucleotide is operably linked to a plant polyadenylation sequence.

9. The method of claim 1, further comprising, prior to said transforming step, engineering said polynucleotide to maximize expression in said plant cell, said engineering comprising determining favored codon usage in said plant cell and altering said polynucleotide by increasing the frequency of favored codons.

10. A method of increasing biomass, increasing total protein in seeds and plants, increasing total carbon/nitrogen level, increasing grain density, or increasing plant yield comprising culturing a plant comprising transgenic cells that comprise a polynucleotide encoding a polypeptide having glutamate dehydrogenase activity under conditions where said polynucleotide is expressed in said cells, whereby biomass is increased, total protein in seeds and plants is increased, total carbon/nitrogen level is increased, grain density is increased, or plant yield is increased, as compared to an untransformed plant.

12. The method of claim 10, wherein said polypeptide is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 24, SEQ ID NO: 26, and fragments thereof having glutamate dehydrogenase activity.

13. A plant produced by the method of claim 2, said plant having increased or decreased nitrogen metabolism as compared to an untransformed plant.